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Presence of Human Herpesviruses 6, 7, and 8 DNA Sequences in Normal Brain Tissue

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The three novel human herpesviruses (HHV) 6, 7, and 8 are predominantly, but not exclusively, lymphotropic. In an attempt to elucidate their neurotropism in vivo, viral DNA sequences present in fresh autopsy cortical brain tissues obtained from 84 consecutive Chinese subjects (mean age, 66.9 years; range, 21–98 years) were detected by a nested polymerase chain reaction. These patients were apparently immunocompetent and free of clinical signs of viral diseases. HHV-6 DNA was detected in 36 of 84 (42.9%) patients, and the DNA-positive and -negative groups did not show a significant difference in age or sex distribution. Of the 36 HHV-6 DNA-positive cases, 9 (25%) were variant A and 27 (75%) were variant B. In view of the lower prevalence of variant A than variant B in the adult population, the two variants may share a comparable neuroinvasive potential. HHV-7 and HHV-8 DNA were detected respectively in three and two patients. The low positive rates of HHV-7 and HHV-8 may represent a relatively lower neuroinvasive potential of the viruses. Alternatively, the localization of HHV-7 and HHV-8 may be more restricted and the sampled cortical tissues may not represent the most abundant site of persistence in the nervous system. The results provide molecular evidence of the presence of the three newly identified herpesviruses in brain tissue. The pathogenic role for HHV-7 and HHV-8, as with HHV-6, in neurological diseases should not be overlooked. ***J. Med. Virol. 59:491–495, 1999.***

KEY WORDS: neurotropism; neuroinvasion; persistence; nervous system; PCR

INTRODUCTION

In the past decade, the family *Herpesviridae* has been expanded to include three new members. Human herpesvirus 6 (HHV-6) was first isolated in 1986 from the peripheral blood lymphocytes of patients with lymphoma associated with AIDS [Salahuddin et al., 1986]. This virus has been recognized as a cause of roseola infantum in infants [Yamanishi et al., 1988], acute nonspecific febrile illness in young children [Pruksananonda et al., 1992], and can give rise to a mononucleosis-like syndrome in adults [Akashi et al., 1993]. Cases of fulminant hepatitis, hemophagocytic syndrome, pneumonitis, myelosuppression, and encephalopathy associated with HHV-6 infection have also been reported [Braun et al., 1997; Levy, 1997]. Human herpesvirus 7 (HHV-7) was first isolated in 1990 from the activated T-cells of a healthy individual [Frenkel et al., 1990]. The disease association of HHV-7 is less well defined. It has been shown that HHV-7 is associated with childhood febrile illnesses, and a pathogenic role of HHV-7 in immunocompromised patients has also been suggested [Levy, 1997]. Human herpesvirus 8 (HHV-8) was identified in 1994 from Kaposi's sarcoma tissue of an AIDS patient and has been associated with Kaposi's sarcoma, body cavity-based lymphoma, and multicentric Castleman's disease [Chang et al., 1994].

While these three newly identified human herpesviruses are predominantly lymphotropic, recent evidence suggests that their tissue tropism in vivo to be more widespread. HHV-6 has been detected in cerebrospinal fluid (CSF) in association with exanthem subitum [Kondo et al., 1993; Suga et al., 1993] and encephalitis [McCuller et al., 1995; Cole et al., 1998]. The virus has also been detected in brain tissue of normal and immunosuppressed individuals [Luppi et al., 1994, 1995], and in oligodendrocytes near demyelinating plaques in patients with multiple sclerosis [Challoner et al., 1995]. HHV-7 shares a high degree of homology with HHV-6 and is classified under the same subfamily

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TABLE I. Primers Used for PCR

Target DNA	Primer	Annealing (°C)	Amplicon (bp)	Reference
HHV-6, major capsid protein gene	Outer~H6-6: 5′-AAGCTTGCACAATGCCAAAAAACAG-3′; H6-7: 5′-CTCGAGTATGCCGAGACCCCTAATC-3′	55	223	Gopal et al. [1990]
	Inner~NH-6: 5′-TCCATTATTTTGGCCGCATTCGT-3′; NH-7: 5′-TGTTAGGATATACCGATGTGCGT-3′	55	130	Wang et al. [1996]
HHV-6, large tegument protein gene	Outer~P1: 5′-AGTCATCACGATCGGCGTGCTATC-3′; P2: 5′-TATCTAGCGCAATCGCTATGTCG-3′	58	287	Lyall and Cubie [1995]
	Inner~P3: 5′-TCGACTCTCACCCTACTGAACGAG-3′; P4: 5′-TGACTAGAGAGCGACAAATTGGAG-3′	58	163	
HHV-7	Outer~P1: 5′-TATCCCAGCTGTTTTCATATAGTAAC-3′; P2: 5′-GCCTTGCGGTAGCACTAGATTTTTTG-3′	55	186	Sada et al. [1996]
	Inner~P3: 5′-CAGAAATGATAGACAGATGTTGG-3′; P4: 5′-TAGATTTTTTGAAAAAGATTTAATAAC-3′	55	124	
HHV-8 ORF 26	Outer~KS-1: 5′-AGCCGAAAGGATTCCACCAT-3′; KS-2: 5′-TTCGTGTTGTCTACGTCCAG-3′	55	233	Chang et al. [1994]
	Inner~H8NS1: 5′-ACGGATTTGACCCCGTGTTC-3′; H8NS2: 5′-AATGACACATTGGTGGTATA-3′	56	160	Monini et al. [1996]
HHV-8 ORF 25	Outer~H8P1: 5′-AGGCAACGTCAGATGTGAC-3′; H8P2: 5′-GAAATTACCCACGAGATCGA-3′	55	328	Boshoff et al. [1996]
	Inner~H8P3: 5′-GGAATTATCTCGCAGGTTGCC-3′; H8P4: 5′-CATGGGAGTACATTGTCAGGACCTC-3′	56	213	

β-herpesvirinae. It is therefore important to elucidate whether HHV-7 shares a similar neurotropic property. The detection of HHV-8 in paravertebral ganglia [Corbellino et al., 1996] and CSF [Brink et al., 1998] of patients with AIDS and Kaposi's sarcoma has also led to the question regarding neurotropism of the virus. In this study, the presence of these novel herpesviruses in a series of postmortem brain tissues was investigated.

MATERIALS AND METHODS

Study Samples

A total of 84 consecutive Chinese patients who underwent postmortem were included. A 2-mm^3 block of fresh autopsy brain specimen was obtained from the frontal cortex of each patient. Hemorrhagic areas and areas with visible blood vessels were excluded. Great care was taken to avoid contamination with blood. Stringent procedures were followed to avoid cross-contamination between specimens obtained from different patients. Thorough cleansing of the postmortem room was performed between cases and a new set of cutting instruments was used for each case. The tissues were homogenized and subjected to total DNA extraction using the QIAamp Tissue Kit (QIAGEN, Germany). The quality of DNA present in extracted preparations was assessed by the polymerase chain reaction (PCR) using primers that amplify a 358-bp fragment of the human β-globin gene [Lo et al., 1989]. The study was approved by the local ethics committee.

Viral DNA Detection

The presence of viral DNA was detected by nested PCR using previously published primers (Table I). Briefly, 5 μl of the extracted DNA was amplified in a 50-μl reaction mixture containing PCR buffer (10-mM Tris-HCl, pH 8.3; 50-mM KCl, and 1.5-mM $MgCl_2$), 200 μM of each dNTP, 1 unit of *Taq* polymerase (Pharmacia Biotech, Sweden), and 0.25 μM of each primer. An initial denaturation at 94°C for 4 min was followed by 30 cycles of 1 min each at denaturation (94°C), annealing at temperatures as shown in Table I and extension (72°C), respectively, with an 8-min additional extension step at 72°C after the last cycle. Two microliters of the first-round PCR product was amplified in a second-round PCR using the same conditions with omission of the initial denaturation step. The PCR product was electrophoresed and visualized by ethidium bromide staining. Primers used for HHV-6 DNA amplification have shown to be consensus and carry the same sensitivity in revealing variants A and B [Wang et al., 1996]. To avoid possible contamination of the PCR mixture, all reactions were carried out under stringent conditions following the recommendations of Kwok and Higuchi [1989]. A negative control was included following each fifth sample. In addition, all positive samples were repeated in a separate PCR run and were all reproducible.

Specificity and Sensitivity of PCR

To determine the specificity of PCR, purified DNA from each of the eight human herpesviruses was used as template for PCR amplification. The HHV-6, HHV-7, and HHV-8 nested PCR did not result in cross-amplification from each other and from other human herpesviruses. The lower detection limit of PCR was estimated by limiting dilution using plasmids containing the target sequences of HHV-6, HHV-7, and HHV-8, respectively. These nested PCR assays showed sensitivity equivalent to 5–10 molecules of template.

Confirmation and Characterization of PCR Products

HHV-6 DNA detected was further differentiated into variants A and B by another nested PCR coupled with restriction fragment length analysis using *Hin*dIII as previously described [Lyall and Cubie, 1995]. Variant

A does not contain a *Hin*dIII restriction site, whereas the 163-bp PCR product of variant B will be digested into fragments of 97 and 66 bp. This *Hin*dIII restriction site present at position 2945 of the large tegument protein gene of HHV-6B, but not in HHV-6A, has previously been used to discriminate between the two variants [Lyall and Cubie, 1995; Secchiero et al. 1995]. The specificity of the amplification products of HHV-7 PCR was confirmed by restriction enzyme digestion using *Eco*RI resulting in fragments of 79 bp and 45 bp [Berneman et al., 1992; Wilborn et al., 1995]. For HHV-8, positive samples were confirmed by another nested PCR amplifying a nonoverlapping region at ORF 25 [Boshoff et al., 1996].

Statistical Analysis

The χ^2 test was used to analyse associations between categorical variables. The independent-samples *t*-test was used to analyze differences between numerical variables. All tests were two-tailed and *P* values less than 0.05 were regarded as significant.

RESULTS

The 84 studied postmortem cases were aged from 21 to 98 years (mean, 66.9; SD, 17.4); 54 were male and 30 were female. These patients were apparently immunocompetent and free of clinical signs of viral diseases. Of the 17 patients who died of intracranial hemorrhage, 14 were due to head injuries, two were due to bleeding from pituitary adenoma, and one was as a result of uncontrolled hypertension. The other 67 patients died of causes unrelated to the central nervous system pathology.

Overall, 42.9% (36/84) had HHV-6 DNA sequences detected in the brain tissue sampled. Nine (25%) were HHV-6 variant A, the other 27 harbored variant B. There was no significant difference in the HHV-6 DNA-positive rate between patients who died of intracranial hemorrhage and those who died of diseases unrelated to the central nervous system (50.0 vs. 40.1%; $P = 0.49$ by χ^2 test). When analyzed by age, the HHV-6 DNA-positive and -negative groups did not reveal a significant difference (mean age, 67.3 vs. 66.7 years; 95% CI, -8.3 to 7.1; $P = 0.88$ by *t*-test). Also, the HHV-6 DNA-positive and -negative groups did not reveal any significant difference in the sex ratio (male:female, 1.25:1 vs. 2.4:1; $P = 0.15$ by χ^2 test).

Three patients (aged 42, 67, and 77 years) were positive for HHV-7 DNA and two patients (aged 49 and 75 years) were positive for HHV-8 DNA. All these five patients died of diseases unrelated to the central nervous system.

DISCUSSION

Members of the family *Herpesviridae* are notorious for their ability to persist for the lifetime of infected hosts. Reactivation of herpesviruses may result in a wide spectrum of clinical manifestations ranging from asymptomatic shedding to fatal diseases. The organ from which the virus is reactivated is often a critical determinant of the clinical outcome.

It has been suggested that the two variants of HHV-6 differ in their cellular tropism and potential pathogenicity [Braun et al., 1997; Levy, 1997]. Studies based on peripheral blood mononuclear cells and saliva samples obtained from both immunocompromised and healthy populations have consistently shown that HHV-6B is the predominant variant found in these cell types [Drobyski et al., 1993; Lyall and Cubie, 1995; Braun et al., 1997]. In contrast, the reported proportions of the two variants found in CSF and neural tissues are more variable. Hall et al. [1998] reported that variant A was identified more frequently in CSF than in peripheral blood mononuclear cells from children with primary infection. They also found that in children with dual infection, only HHV-6A persisted in CSF. Luppi et al. [1994] also found that all five HHV-6 DNA-positive samples derived from normal brain tissue harbored variant A. However, McCullers et al. [1995] reported that all three HHV-6 DNA-positive CSF samples obtained from a series of patients with focal encephalitis of unknown etiology were variant B. On the other hand, both variants A [Knox et al., 1995; Bosi et al., 1998] and B [Drobyski et al., 1994; Cole et al., 1998] have been detected in CSF of marrow graft recipients and AIDS patients with encephalitis.

While using PCR to detect viral DNA sequences is highly sensitive, it is also prone to cross-contamination. In this study, considerable precautions were taken to avoid cross-contamination in collecting and transporting the specimens. In addition, the PCR results did not show clustering of positivity that might suggest such contamination. Furthermore, the possibility of cross-contamination between PCR mixture was excluded by the reproducibility of results when original specimens were reamplified with the same and different sets of primers.

The results show that both HHV-6A and B are neurotropic in vivo, which is in line with the in vitro observation that both variants can infect primary astrocytes [He et al., 1996]. In the adult population, HHV-6B is still the major (75%) variant found in cortical brain tissue, although the proportion (25%) of variant A is relatively higher when compared with nonneural tissues. While HHV-6B is ubiquitous in the adult population, the proportion of adults infected with HHV-6A is not well defined and is thought to be much less prevalent than that of variant B [Wang et al., 1999]. Thus, the findings may argue for a comparable neuroinvasiveness of the two variants that persist in brain tissues of a large proportion of the general population.

While HHV-7 DNA has been detected in CSF [Torigoe et at., 1996; Portolani et al., 1998], to the best of our knowledge, this is the first report on the presence of HHV-7 DNA sequences in normal brain tissue. The result indicates that HHV-7, like HHV-6, is neurotropic, which is in line with the reports of two previously healthy children with primary HHV-7 infection who were complicated with acute hemiplegia [Torigoe et al.,

1996], and a marrow graft recipient with rash and encephalitis associated with HHV-7 infection [Chan et al., 1997]. The finding of a low HHV-7 DNA-positive rate (3/84) may reflect its lower neuroinvasiveness when compared with HHV-6. Alternatively, the distribution of HHV-7 in the nervous system may be more restricted and our sampled cortical tissue may not represent the most abundant site of viral persistence.

Data on the distribution of HHV-8 in the general population have been limited by the lack of an efficient assay. At present, the reported seroprevalence in adults varies from 1% to 50% [Lennette et al., 1996; Goedert et al., 1997; Mayama et al., 1998]. Whether our result of 2/84 of the sampled brain tissues being positive for HHV-8 DNA represents a significant proportion of the infected individuals requires further study. Nevertheless, the potential role of HHV-8 in neurological diseases should not be neglected.

The results provide molecular evidence of the presence of the three novel members of the herpesvirus family, HHV-6, 7, and 8, in normal brain tissue. This raises many interesting questions as to where these viruses are located, the routes of entry, their latency states and associations with brain pathology.

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